Placenta growth factor induces 5-lipoxygenase activating protein to increase leukotriene formation in sickle cell disease

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Abstract

Individuals with sickle cell disease (SCD) have increased inflammation, a high incidence of airway hyper-reactivity (AH) and increased circulating leukotrienes (LT). We show that expression of 5-lipoxygenase and 5-lipoxygenase activating protein (FLAP), key catalytic molecules in the LT pathway, were significantly increased in peripheral blood mononuclear cells (MNC) in patients with SCD, compared to healthy controls. Placenta growth factor (PIGF), elaborated from erythroid cells, activated MNC and THP-1 monocyctic cells to induce LT production. PIGF-mediated increased FLAP mRNA expression occurred via activation of PI-3 kinase, NADPH-oxidase and HIF-1α. HIF-1α siRNA reduced PIGF-induced FLAP expression. FLAP promoter-driven luciferase constructs demonstrated that PIGF-mediated luciferase induction was abrogated upon mutation of HIF-1α response element (HRE), but not the NF-κB site in the FLAP promoter; a finding confirmed by ChIP analysis. PIGF also increased HIF-1α binding to the HRE in the FLAP promoter. Therefore, it is likely that the intrinsically elevated levels of PIGF in SCD subjects contribute to increased LT, which in turn, mediate both inflammation and AH. Herein, we identify a mechanism of increased LT in SCD, and show HIF-1α as a hypoxia-independent target of PIGF. These studies provide new avenues to ameliorate these complications.
Introduction

Inflammation is increasingly recognized as central to the pathophysiology of sickle cell disease (SCD), and is manifest as leukocytosis, elevated levels of inflammatory cytokines, and activation of neutrophils, monocytes, and endothelial cells.\textsuperscript{1-4} It is present at steady state and is strongly associated with acute painful events, acute chest and early mortality.\textsuperscript{5,6} Current evidence strongly suggests that inflammation contributes to the endothelial cell dysfunction, potentiates vaso-occlusion and may also give rise to the airway hyperreactivity (AH) that often accompanies SCD.\textsuperscript{7-10} Also intriguing is the spectrum of lung disease seen in this patient population, which spans from an increased incidence of AH and obstructive lung disease in children,\textsuperscript{11-13} to restrictive lung disease and pulmonary vascular remodeling, which is associated with pulmonary hypertension in adults.\textsuperscript{14-18}

Leukotrienes (LT) mediate both inflammation and AH.\textsuperscript{19-22} Five lipoxygenase (5-LO) and its activating partner, five lipoxygenase activating protein (FLAP) catalyze the production of LT from arachidonic acid (AA) by generating 5-hydroperoxyeicostatraenoic acid (5-HPETE) and leukotriene A\textsubscript{4} (LTA\textsubscript{4}). LTA\textsubscript{4} is the pivotal intermediate from which other LT i.e. LTB\textsubscript{4} and cysteinyln LT (CysLT) - LTC\textsubscript{4}, LTD\textsubscript{4}, and LTE\textsubscript{4} are formed.\textsuperscript{20} LTB\textsubscript{4} is one of the most potent chemoattractant for neutrophils, eosinophils and mediator of inflammation. CysLT, on the other hand, are potent bronchoconstrictors that play an important role in edema, inflammation and mucus secretion in asthma, and were previously termed “slow releasing substances”.\textsuperscript{23} LT play an important role in the pathogenesis of inflammatory disorders, specifically asthma, rheumatoid arthritis and inflammatory bowel disease.\textsuperscript{19-21} Studies by Bigby and coworkers\textsuperscript{24,25} have shown that both TNF-\alpha and LPS induce the expression of FLAP in THP-1 cells. These studies showed the importance of NF-kB and C/EBP transcription factors in the LPS mediated FLAP expression.\textsuperscript{24}
LTB₄ levels are higher in SCD patients at steady state, which are further increased in vaso-occlusive pain crises (VOC) and acute chest syndrome (ACS). Very recently, increased LTE₄ has been observed in patients with SCD, which is associated with a higher incidence of pain. However, relatively less is understood about how LT are increased in SCD at the molecular level.

Placenta Growth Factor (PlGF) is an angiogenic growth factor with similar effects on endothelium as VEGF, and is primarily expressed by placental trophoblasts. More recently, we and others show that erythroid cells, but not other hematopoietic cells, produce PlGF and its expression is high in SCD and thalassemia. VEGFR1 is its cognate receptor, which is expressed on endothelial cells, alveolar epithelial cells, mast cells and monocytes. We have previously shown that plasma levels of PlGF are high in SCD patients compared to control, which correlated well with SCD severity. Moreover, we showed that mononuclear cells (MNC) of SCD patients were in activated state as demonstrated by increased levels of cytochemokines (IL-1β, IL-8, MCP-1 and VEGF) as compared to healthy controls. Treatment of MNC from healthy individuals with PlGF in vitro increased expression of the same cytochemokines as was seen in SCD, strongly suggesting that PlGF may contribute to increased cytochemokine expression from monocytes. The cytochemokines induced by PlGF are potent leukocyte activators and chemo-attractants. Injection of a PlGF-adenovirus vector causes leukocytosis in mice. These data suggest PlGF may contribute to leukocyte activation and leukocytosis in vivo. Conceivably, increased leukocytosis and leukocyte activation in SCD would lead to amplified levels of LT, resulting in AH. We hypothesized that the chronic inflammation and increased incidence of AH in patients with SCD could be explained by the activation of monocytes by PlGF to induce LT production.
In the present study, we show that MNC from SCD subjects at steady state show significantly increased expression of 5-LO and FLAP mRNA, both key catalytic components of the LT pathway, as compared with healthy controls. In addition, PIGF increased FLAP mRNA expression and LT production from peripheral blood monocytes (PBM) and THP-1 monocytic cells. We find that PIGF activated PI-3 kinase, NADPH-oxidase and hypoxia-inducible factor (HIF-1α) to increase FLAP expression. Our studies show, for the first time, to the best of our knowledge, a hypoxia-independent role of HIF-1α in the regulation of FLAP and define some of the mechanisms behind the non-erythroid phenotypic presentations of SCD, and open new avenues for targeted therapeutic approaches.
Materials and Methods

Cells and reagents

All blood samples were obtained from children with homozygous SCD at steady state at their elective clinic appointment with routine clinical blood draws, using IRB approved protocols at Cincinnati Children’s Hospital and with informed consent in accordance with the Declaration of Helsinki. Peripheral blood monocytes were isolated from EDTA blood from healthy volunteers after obtaining informed consent according to a protocol approved by the IRB at USC-LAC Hospital. To ensure values represent true steady state, samples were obtained from patients who had no acute sickle events, fever, infections 3 weeks prior, or 3 weeks after the blood draw and were not transfused within the last 90 days. Complete blood counts on the SCD patients revealed a WBC count of 11,600±1,400/µL, hemoglobin of 9.5±0.4g/dL, and platelets 466,000±63,400/µL. The proportion of eosinophils in the WBC fraction, and the absolute eosinophil count (340±90; n=9) were normal; and reticulocytes were elevated, as expected (8.5±1.7%). Complete blood counts were not performed on normal controls. Four of nine patients were on hydroxyurea. MNC were isolated as described, resuspended in RPMI-1640 medium and treated with PlGF (250ng/ml) for 24hr. THP-1 cells (ATCC; Manassas, VA) were cultured in RPMI containing 10% FBS and were placed in serum-free RPMI overnight prior to PlGF treatment.

Reagents were obtained as follows: PlGF (R&D Systems, Minneapolis, MN), LY294002, Diphenyleneiodonium chloride (DPI), PD98059, SB203580 and SP600125 (Tocris Bioscience, Ellisville, MO), R59949 and PDTC (Calbiochem, Gibbstown, NJ), MK866 and U73122 (Biomol International Inc, Plymouth Meeting, PA), 32P-UTP (MP Biomedicals, Solon, OH), antibodies against VEGFR1, HIF-1α, HIF-1β, FLAP, prolyl hydroxylase-2 (PHD-2), β-actin and secondary antibodies conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA). The full length -3368FLAP-pGL3 promoter construct and deletion constructs (−965FLAP-pGL3, −371FLAP-pGL3
and -134-FLAP-pGL3) were generously provided by Dr. Timothy Bigby (VA Hospital San Diego, La Jolla, CA). The HRE-Luc (Dr. Michael Kahn), PTEN plasmid (Dr. Debbie Johnson) and HIF-1α and HIF-1β expression plasmids (Dr. Michael Stallcup) were obtained from investigators at USC Keck School of Medicine, Los Angeles, CA. HIF-1α siRNA, HIF-1α scRNA, PHD-2 siRNA and PHD-2 scRNA were synthesized at the Microchemical Core facility of USC Norris Comprehensive Cancer Center. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). All other reagents, unless otherwise specified, were purchased from Sigma (St Louis, MO).

mRNA analysis

Total RNA was isolated using TriZOL reagent (Invitrogen, Carlsbad, CA). Ribonuclease protection assay (RPA) was performed using a custom Riboquant Multi-probe template comprised of 5-LO, FLAP, HIF-1α and GAPDH (BD Biosciences, San Diego, CA) as described. The intensity of the bands was analyzed using the Spot-Denso software on the Alpha Imager 2000 gel documentation system (Alpha Innotech Corporation, San Leandro, CA). Real-time PCR (qPCR) was carried out using the iScript One-Step RT-PCR kit with SYBR Green as per manufacturer’s instructions (Bio-Rad, Hercules, CA) on ABI PRISM 7900 (Applied Biosystems, Foster City, CA). Briefly, 40 cycles of amplification was carried out following reverse transcription at 95°Cx10s and 60°Cx30s, utilizing primers listed in Table 1. Relative quantification (RQ) values of 5-LO and FLAP mRNA expression were calculated as $2^{\Delta\Delta C_t}$ by the comparative C_t method. Where $\Delta\Delta C_t = (C_t$ target gene of SCD sample – $C_t$ GAPDH of SCD sample) - (C_t target gene of control sample - $C_t$ GAPDH of control sample).

Leukotriene assay

THP-1/PBM (1.5×10^6 cells/ml) were treated with PIGF (250 ng/ml) for 24hr and supernatants were assayed for LT utilizing ELISA kit (Cayman Chemical, Ann Arbor, MI).
Estimation of intracellular reactive oxygen species (ROS)

Briefly, THP-1 (2×10^6 cells) were washed with PBS, incubated with 5µM 2’,7’-dichlorofluorescein-diacetate dye in RPMI at 37°C for 30min in the dark, then washed thrice with PBS to remove excess dye, and stimulated with PIGF for 4hr. Fluorescence intensity was analyzed for 60sec using RF-551 spectrofluorometric detector (Shimadzu, Kyoto, Japan) with excitation at 488nm and emission at 525nm. THP-1 cells loaded with the dye for 30min were used as a blank control.

Western blot analysis

The cytosolic and nuclear extracts were prepared from THP-1 cells as described. Briefly, 5x10^6 cells were washed and resuspended in 400µl of cell-lysis buffer for 20min. The homogenate was centrifuged at 10,000g for 30sec and cytosolic supernatant was collected. The nuclear extract was obtained by resuspending nuclear pellet in 50µl of nuclear extraction buffer on ice for 60min. The cytosolic extracts were used to analyze FLAP and PHD-2 protein while nuclear extracts were subjected to HIF-1α and HIF-1β analysis. The protein bands were detected with Immunobilon western reagents (Millipore Corporation, Billerica, MA).

The mutagenesis of human FLAP promoter

Mutant constructs of the human FLAP-Luc promoter were generated using wild type -371 FLAP-Luc construct as a template by the Quick Change site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) using the primers shown in Table 1. The double mutant of HIF-1α binding sites, represented as HRE-M1+2 was generated using HRE-M1 as a template. Mutations were confirmed by sequencing.
**Transient transfection**

THP-1 cells (1X10^6) were transfected with various siRNA constructs (50nM) and luciferase reporter plasmids by nucleofection with Nucleofector kit-V (Amaxa Biosystems, Cologne, Germany). The sense and antisense siRNA oligonucleotides were annealed as described. The β-galactosidase plasmid (0.5μg) was co-transfected with reporter constructs (0.5μg) to control for transfection efficiency. Post-tranfection, the cells were kept in complete medium overnight, serum free medium for 3hr and treated with PlGF for the indicated times. The cells were lysed and analyzed for luciferase activity and β-galactosidase activity using kits (Promega, Madison, WI). Luciferase values were normalized to β-galactosidase values. Data are expressed relative to the activity of the promoter-less pGL3 basic vector.

**Electrophoretic mobility shift assay (EMSA) for transcription factor HIF-1α**

The single stranded oligonucleotides (Table 1) were biotin labeled using a Lightshift Chemiluminescent EMSA kit (Pierce, Rockford, IL) and the complementary strands were annealed in equimolar ratios for 1hr at 37°C. The DNA binding reaction included nuclear protein extract (5μg), 5% glycerol, 5mM MgCl_2_, 50ng/μl poly(dI·dC), 0.05% NP-40 and 0.5ng biotinylated probe and incubated at room temperature for 20min. The specificity of DNA-protein interaction was demonstrated using 50-fold excess of unlabeled probe. For supershift assays, nuclear extracts were pre-incubated for 1hr on ice with HIF-1α antibody (2μg). The samples were then subjected to non-denaturing 6% polyacrylamide gel electrophoresis in 0.5×TBE, transferred to a Hybond-N* nylon membrane (Amersham Biosciences, Piscataway, NJ) followed by detection with streptavidin-HRP/chemiluminescence.
Chromatin immunoprecipitation (ChIP) assay

THP-1 (10x10⁶ cells) were treated with PI GF in serum-free RPMI for the indicated time periods in the presence/absence of inhibitors. ChIP analysis was performed utilizing HIF-1α antibody as previously described. Briefly, formaldehyde fixed cells were lysed and chromatin was sheared by sonication (6x15sec, 40% potency). The lysates were pre-cleared for 2hr at 4°C with Protein A-Sepharose beads. Immunoprecipitation was carried out at 4°C overnight with HIF-1α antibody or control IgG antibody. Protein-A immune complexes were collected and washed sequentially with low salt buffer, high salt buffer and TE buffer. DNA cross-links were reversed at 65°C overnight, DNA was extracted by phenol/chloroform/isoamyl alcohol and then ethanol-precipitated. Immunoprecipitated DNA was subjected to PCR amplification for 30 cycles under the following conditions: 95°C for 30s, 58°C for 60s and 72°C for 120s, utilizing primers shown in Table 1. The PCR products were subjected to agarose gel electrophoresis followed by densitometric analysis. The values were normalized to input DNA.

Statistical Analysis

Control and PI GF treated cells were compared by Student’s t-test. One-way ANOVA followed by Turkey-Kramer test was used for multiple comparisons using the Instat-2 software program (GraphPad, San Diego, CA). Values of p<0.05 were considered statistically significant.

Results

MNC of SCD subjects express increased levels of 5-LO and FLAP mRNA at steady state

We first examined whether expression of 5-LO and FLAP, key regulatory enzymes in LT formation, were affected in MNC from SCD subjects. As shown in Figure 1A, there was approximately 3-fold increase in 5-LO mRNA expression in SCD compared to healthy controls.
by qRT-PCR (ΔCt, 23.12±6.49 vs. 7.65±1.5; n=9; p<0.05). Additionally, there was a 3.8-fold increase in FLAP mRNA expression in SCD compared to healthy controls (ΔCt, 36.0±7.25 vs. 9.42±2.12; n=9; p<0.013) as shown in Figure 1B. There was also approximately 3-fold relative change in RQ values of 5-LO and FLAP mRNA expression in SCD compared to control subjects (Figure 1C). PIGF concentrations in the plasma of the same samples were significantly higher in SCD patients (24.7±1.6 pg/mL, n=9) than in control (13.7±0.4; n=9; p<0.001), consistent with previously reported values.31

PIGF induced FLAP mRNA expression in THP-1 and PBM cells

THP-1 cells showed maximal increase in FLAP mRNA at 250ng/ml, as previously observed for expression of cytochemokines,33 therefore 250ng/mL PIGF was utilized in all further experiments (Figure S1). The FLAP mRNA expression peaked at 24 hr (Figure S2). Although this in vitro PIGF concentration utilized herein are similar to those previously reported,43,44 they are much higher than the estimated levels of plasma PIGF in SCD patients. However, these concentrations may not reflect the local concentrations of PIGF in the microenvironment to which the cells are exposed, as seen with other hematopoietic growth factors. Besides, PIGF has different splice variants, some with high basic residues with heparin/matrix binding activity; where it may be presented to cells directly at high local concentrations. The ELISA used to estimate plasma PIGF only detects free circulating PIGF and may under-estimate the concentration presented to cells.

Next, we determined the time course of FLAP induction. As shown in Figure 1D, PIGF treatment of THP-1 cells resulted in a time dependent (6, 12 and 24hr) increase in mRNA expression of FLAP, as determined by RPA. There was 4.5-fold (458±34%) increase in FLAP mRNA expression at 24hr in THP-1. Additionally, polymyxin B (5µg/mL), an inhibitor of endotoxin, did
not inhibit PIGF induced FLAP mRNA expression (data not shown). Moreover, PIGF treatment of PBM from healthy controls showed approximately 3.6-fold (365±6%) increase in FLAP mRNA expression (Figure 1D). Since both THP-1 and PBM cells were responsive to PIGF in up regulating FLAP expression, we utilized THP-1 monocytic cells as a model system for ease of culturing and transfection for further mechanistic studies.

**PIGF causes release of LT from THP-1 cells and PBM**

Treatment of THP-1 cells with PIGF for 24hr, resulted in a 4-fold (414±13.4%) increase in the release of LTE₄, as determined by ELISA (Figure 2A). However, these cells did not release LTB₄, as has been previously observed.⁴⁵ Moreover, pretreatment of THP-1 cells with an antibody to VEGFR1 or pharmacological inhibitors for PI-3 kinase (LY294002), NADPH-oxidase (DPI), FLAP (MK866) and phospholipase C (PLC) (U73122) reduced PIGF mediated LTE₄ release by 95±4%, 78±9%, 88±4%, 94±4%, and 97±5% respectively (Figure 2B). Leukotriene agonists can release arachidonic acid upon receptor activation followed by cPLA₂ activation within seconds. However, we did not observe an effect on LTE4 production at early time points (Figure S3). PIGF also caused a 4-fold (423±22%) increase in the release of LTE₄ from PBM (Figure 2A).

**PIGF induced FLAP expression involves activation of PI-3 kinase, NADPH oxidase and HIF-1α**

As shown in Figure 2C, PIGF induced FLAP mRNA expression was inhibited by VEGFR1 antibody (93±4%), LY294002 (75±3%), DPI (98±4%) and R59949 (84±7%), the latter being a putative inhibitor of HIF-1α. However, inhibitors of NF-κB (PDTC), p38MAP kinase (SB203580) and MAP kinase (PD98059) did not inhibit PIGF-induced FLAP expression. Since DPI inhibited
FLAP expression, we determined whether PIGF increased the formation of ROS in THP-1 cells. As shown in Figure 2D, PIGF caused a 6-fold (598±54%) increase in ROS formation, which was attenuated by VEGFR1 antibody (71±9%) and DPI (57±2%), while LY294002 and SB203580 did not affect ROS formation. These results indicate that PIGF binding to VEGFR1 causes activation of NADPH-oxidase to generate ROS, which is independent of the PI-3 kinase pathway.

We wanted to determine whether a similar effect of PIGF can be seen on the FLAP protein levels. As shown in Figure 3A, PIGF caused a time dependent (6-24hr) increase in FLAP protein expression. There was a 2.6-fold (266±9%) increase in FLAP protein at 24hr, which was significantly reduced by LY294002 and DPI by ~80%. Taken together, PIGF mediated FLAP expression involves activation of PI-3 kinase and NADPH oxidase.

**PIGF induced HIF-1α mRNA and protein expression**

It is well established that hypoxia increases HIF-1α protein levels but not HIF-1α mRNA.\textsuperscript{46} In contrast, we observed that PIGF treatment of THP-1 cells increased HIF-1α mRNA expression in a time dependent manner (6-24hr) (Figure 1D). There was a 3-fold (299±8%) and a 2.5-fold (250±5%) increase in HIF-1α mRNA levels in THP-1 and PBM, respectively, at 24hr. As shown in Figure 2C, both VEGFR1 antibody and LY294002 reduced PIGF induced HIF-1α mRNA expression by ~80%, while DPI, R59949, PDTC, SB203580 and PD98059 did not affect HIF-1α mRNA levels. Although the ROS inhibitor, DPI, attenuated FLAP mRNA expression (Figure 2C, lane 5), it did not reduce HIF-1α mRNA; therefore, we determined whether ROS exerted its effect on HIF-1α protein levels. As shown in Figure 3B, PIGF led to a time dependent (6-24hr) increase in HIF-1α protein levels in nuclear extracts of THP-1 cells, which was attenuated by
LY294002 and DPI by ~80%. It is pertinent to note that HIF-1β, a constitutive protein, remained unchanged, showing a specific induction of HIF-1α in response to PIGF (Figure 3B). These results indicate that PIGF mediated activation of PI-3 kinase leads to increases in both HIF-1α mRNA and protein, while ROS formation through NADPH oxidase activation contributes to HIF-1α protein stabilization.

**PIGF mediated FLAP mRNA expression and LTE₄ release requires HIF-1α**

PIGF treatment of THP-1 cells transfected with HIF-1α siRNA resulted in reduction of both HIF-1α (87±5%) and FLAP (78±3%) mRNA expression (Figure 4A, lane 3). However, scrambled (sc) HIF-1α siRNA did not affect HIF-1α and FLAP mRNA levels (Figure 4A, lane 4). In addition, HIF-1α siRNA attenuated PIGF mediated LTE₄ release by 88±4%, while scHIF-1α siRNA had no effect (Figure 4B). It is pertinent to note that transfection with HIF-1α siRNA but not scHIF-1α siRNA reduced HIF-1α protein levels (Figure 4C). Furthermore, overexpression of HIF-1α led to a 3-fold increase in FLAP mRNA in the absence of PIGF treatment (Figure 4A, lane 5). However, HIF-1β overexpression did not change FLAP mRNA expression (Figure 4A, lane 6). Moreover, PHD-2 siRNA increased HIF-1α protein by inhibiting its degradation, resulting in a 4-fold (395±8%) increase of FLAP mRNA expression in response to PIGF (Figure 4A, lane 7) compared to cells transfected with scPHD-2 siRNA (102±8%; lane 8), supporting the role of HIF-1α in FLAP expression. Recent studies have shown that TGF-β1 induces HIF-1α stabilization through selective inhibition of PHD-2 protein levels in HepG2 cells. Therefore, we analyzed the effect of PIGF on PHD-2 protein levels. As shown in Figure 4D, PIGF treatment of THP-1 cells for 6, 12 and 24hr did not change PHD-2 protein levels. Taken together, these data indicate that PIGF mediated FLAP expression and LTE₄ release involves HIF-1α.
PIGF augments hypoxia-response element (HRE) promoter activity

Since PIGF mediated FLAP expression was attenuated by HIF-1α siRNA, we investigated whether this occurred via HREs, which are present in promoter regions of several genes regulated by HIF-1α. As shown in Figure 5A, PIGF caused a 5-fold increase in HRE-Luc activity, which was inhibited by LY294002 (53±5%) and transfection with PTEN (79±4%). In addition, transfection with HIF-1α siRNA reduced HRE-Luc activity by 77±3%, while scHIF-1α siRNA had no effect (Figure 5B). Moreover, HIF-1α overexpression showed a 5.5-fold increase in HRE-Luc activity, in the absence of PIGF. In contrast, overexpression of HIF-1β did not change HRE-Luc activity. These data show that PIGF induced HIF-1α expression can activate HRE.

PIGF mediated FLAP promoter activity requires HRE but not NF-κB binding site

Bigby and coworkers have shown that LPS induced FLAP expression in THP-1 cells required the binding of both NF-κB and C/EBP to its promoter. As shown in Figure 5C, PIGF increased FLAP promoter activity by 5-fold (designated as 100% compared to the promoter less pGL3 vector). Analysis of serial deletion constructs of FLAP promoter showed that -371/+12bp region of the FLAP promoter had similar activity as the full length FLAP promoter, but -134/+12bp promoter construct (lacking the HRE sites) showed reduced activity in response to PIGF (Figure 5C). Thus, we utilized -371/+12bp construct for further studies. This region of the FLAP promoter (Figure 5D) contains two putative consensus HRE (RCGTG) at -170 to -167bp and -251 to -248bp, one NF-κB binding site located at -43 to -34bp and two C/EBP consensus sites located at -36 to -28bp and -25 to -12bp, relative to transcriptional start site. We generated HRE mutant constructs of FLAP promoter, designated as HRE-M1 and HRE-M2 as indicated in Figure 5D. Additionally, we utilized HRE-M1 as a template to generate a construct having mutations in both HRE sites designated as HRE-M1+2. As shown in Figure 5E, tranfections with
HRE-M1 and HRE-M2 showed reduced promoter activation by 66±2% and 40±3%, respectively. However, HRE-M1+2 showed significantly higher inhibition (81±3%) in response to PIGF stimulation. In contrast, two different mutations of NF-κB site (NFkB-M1 and NFkB-M2) did not reduce PIGF mediated FLAP promoter activity. The role of HIF-1α in regulating FLAP promoter activity was also confirmed in PBM by utilizing HRE-M1+2 mutant construct of FLAP promoter. As shown in Figure 5F, PBM transfected with -371FLAP promoter showed a 3.5-fold induction while HRE-M1+2 mutant FLAP promoter resulted in significant attenuation of promoter activity. It is pertinent to note that FLAP promoter construct (-134/+12bp), lacking HRE sites, showed only a 50% reduction in PIGF mediated luciferase activity compared to -371/+12bp construct (Figure 5C), indicating a possible involvement of C/EBP sites in regulating FLAP promoter activity.

Next, we examined the role of PI-3 kinase and HIF-1α in FLAP promoter activity. As shown in Figure 5G, PIGF induced -371FLAP promoter activity was reduced by LY294002 (44±3%) but not by PD98059. Moreover, HIF-1α siRNA inhibited promoter activity by more than 80%, while scHIF-1α siRNA had no effect. Additionally, overexpression of HIF-1α but not HIF-1β resulted in a 5-fold increase in FLAP promoter activity, independent of PIGF stimulation. Taken together, these results suggest that PIGF mediated FLAP promoter activation requires both HRE sites but not NF-κB site located within the first 371bp of the FLAP promoter.

PIGF induces HIF-1α binding in vitro (EMSA) and in vivo (ChIP) to FLAP promoter

To further substantiate whether HIF-1α binds to HRE in the FLAP promoter, we utilized both wild type and mutant oligonucleotide flanking HRE site corresponding to -170 to -167bp region as probes for EMSA (Table 1). As shown in Figure 6A, nuclear extracts of PIGF treated cells...
showed increased HIF-1α DNA binding compared to untreated cells. Moreover, 50-fold excess unlabeled probe competed out HIF-1α DNA binding (lane 3). Additionally, HIF-1α antibody supershifted the band indicating the specificity of HIF-1α DNA binding (lane 4). Furthermore, mutant HRE oligonucleotide showed negligible HIF-1α DNA binding (lane 5) as compared to wild type probe (lane 2) in nuclear extracts of PIGF treated cells. These results were also confirmed by ChIP analysis showing HIF-1α binding to the FLAP promoter in the native chromatin of THP-1 cells. PIGF treated cells showed a 3-fold increase in expected PCR product size of 319bp, corresponding to the FLAP promoter region (-310 to + 9bp) containing two HRE sites (Figure 6B), using primers listed in Table 1. Pretreatment with both LY294002 and DPI reduced the expected PCR product by ~80%. As shown in the middle panel, the amplification of input DNA before immunoprecipitation was equal in all samples. Immunoprecipitation of chromatin samples with control rabbit IgG did not show any amplification (Figure 6B, lower panel). These data indicate that PIGF increases HIF-1α binding to FLAP promoter to upregulate the expression of FLAP in vivo.
Discussion

In this report, we showed that SCD patients at steady state expressed increased levels of 5-LO and FLAP mRNA in MNC, compared to healthy controls. Our studies further showed that PIGF upregulated FLAP mRNA to generate LT from PBM and THP-1 cells. A salient feature of SCD is inflammation, manifest as leukocytosis that occurs in the absence of acute infection or inflammation.5,6 It has been a dilemma to understand how a genetic defect in sickle RBC would result in leukocytosis and leukocyte activation at baseline.2,7,31 Moreover, this leukocytosis correlates well with severity of disease and early mortality in two large studies5,6, although a recent study did not find relationship.48 Also seen is increased AH in children9,11,49 and infants50 in SCD. Moreover, LTB426,51 and LTE427 levels are found to be increased in SCD at baseline, which further increased during episodes of VOC and ACS.26 Herein, we determined whether PIGF played a role in activation of monocytes to generate increased levels of LT, which would contribute to inflammation and asthma in SCD.

Styles and colleagues have shown increased levels of secretory phospholipase A2 (sPLA2) in association with development of ACS in SCD.52 sPLA2 releases AA from membrane phospholipids and has been implicated in the ACS.52,53 Increased AA can be acted upon by 5-LO and FLAP, resulting in production of LTB4 and CysLT. Since PIGF levels are highly elevated in ACS in SCD31; sickle monocytes are activated to generate cytochemokines31,33, PIGF may play a role in ACS by contributing to both inflammation and AH.

Next, we examined the cellular signaling pathway for PIGF-mediated increased FLAP expression and LT release. We observed that PIGF-mediated FLAP expression and LTE4 release was attenuated by antibody to VEGFR1 and inhibition of PI-3 kinase, NADPH-oxidase
and HIF-1α. PIGF mediated LTE₄ release was also inhibited by inhibitors of FLAP (MK866) and PLC (U73122). Since U73122 inhibited the LTE₄ release, we examined if PIGF resulted in LTE₄ release at early time points, and found no effect (data not shown). Thus, it is possible that U73122 inhibits LTE₄ release at 24hr by inhibiting 5-LO, as has been previously reported. PIGF mediated increase in HIF-1α mRNA occurred via activation of PI-3 kinase pathway, but not the NADPH-oxidase pathway. In contrast, activation of the NADPH-oxidase pathway increased HIF-1α protein levels, indicating that HIF-1α protein is stabilized by ROS pathway, as has been previously noted. However, the mechanism(s) by which PIGF stabilizes HIF-1α protein in a hypoxia-independent manner remain unknown. Recent studies have shown that TGF-β1 induces HIF-1α stabilization through selective inhibition of PHD-2, while nitric oxide mediated HIF-1α degradation involves PHD-2 activation, indicating the role of PHD-2 in HIF-1α stabilization. However, we did not observe any change in PHD-2 protein levels in response to PIGF, suggesting that PIGF mediated ROS generation could conceivably inhibit PHD-2 enzyme activity by modifying the levels of various intracellular cofactors such as ascorbate, Fe²⁺ and succinate, and thus stabilize HIF-1α.

Previous studies have shown that LPS induced FLAP expression in THP-1 cells required the binding of both NF-κB and C/EBP to its promoter and deletion analysis of the full length FLAP promoter (-3368/+12bp; accession # 60470) revealed that the first 134bp of the promoter (-134/+12bp) were sufficient for TNF-α and LPS induced FLAP promoter activity. Our studies of deletion constructs of the FLAP promoter showed that -371/+12bp region was equally effective as the full length FLAP promoter in mediating the response to PIGF. Moreover, this effect was mediated via two HRE sites, unlike previously characterized NF-κB site in FLAP promoter. Since mutations of HRE sites in the proximal promoter of FLAP (-371/+12bp) showed residual
promoter activity, we cannot rule out the activation of the C/EBP sites in the FLAP promoter by PIGF.

We confirmed our results by different approaches: we show that PIGF causes a 5-fold increase in HRE-Luc activity, which was abrogated by HIF-1α siRNA. Mutation of either HRE sites in FLAP promoter (HRE-M1 and HRE-M2) attenuated PIGF-mediated FLAP expression. Additionally, mutation of both HRE (HRE-M1+2) resulted in a marked reduction in FLAP promoter activity. However, mutations of the NF-κB site in the promoter had no significant effect. We also show that silencing with HIF-1α siRNA attenuated PIGF-mediated FLAP promoter activation and downstream gene expression. Conversely, overexpression of HIF-1α, but not HIF-1β, augmented FLAP promoter activity and mRNA expression in the absence of PIGF. Overall, our results suggest that PIGF-mediated FLAP promoter activation specifically required HRE, but not the NF-κB sites. EMSA analysis showed increased HIF-1α binding to HRE in the FLAP promoter, in response to PIGF. Additionally, ChIP analysis confirmed the HIF-1α binding to FLAP promoter to upregulate FLAP expression in vivo.

Notably, PIGF may have pleiotropic effect through multiple pathways in SCD, besides increasing LT. We have shown that PIGF increases cytochemokine release from MNCs, which likely contributes to inflammation. This is supported by the evidence that PIGF knockout mice display blunted inflammatory response. We recently showed that PIGF induces expression of endothelin-1 from endothelial cells and endothelin-B receptor in monocytes, molecules that likely contribute to the pulmonary artery hypertension seen in SCD. Notably, CysLT generated by platelet-leukocyte interactions can also stimulate endothelial cells to release von Willebrand factor, which may further potentiate vascular occlusion. Besides their effect on airways and smooth muscle, LT also promote adhesion of neutrophils to endothelial cells and the neutrophil-derived LTA₄ may contribute to the pool of LT. Thus, LT may
contribute to vascular occlusion and reactive air way disease in SCD. Recently, sickle mice have been shown to have an exaggerated propensity to experimentally-induced asthma. These results would be important to confirm in vivo. We are currently investigating the role of PIGF and the 5-LO pathway in inflammation, acute vascular occlusions, ACS and AH in a prospective clinical study; and confirming the direct effects of PIGF in mouse models that simulate the chronic PIGF over-expression in SCD.

In conclusion, our studies show that PIGF, intrinsically released from erythroid cells caused activation of leukocytes, particularly monocytes, to generate increased levels of LT. We showed that the binding of PIGF to VEGFR1 led to activation of PI-3 kinase, NADPH-oxidase and HIF-1α. These data provide a novel mechanism of PIGF-induced FLAP expression which involves activation of HIF-1α in a manner that is independent of hypoxia. Thus, PIGF may provide a link between increased formation of LT, inflammation, vaso-occlusion and AH seen in SCD subjects. Antagonists of the LT pathway, such as LT receptor antagonists, or zileuton, which are currently licensed for asthma, could be utilized to ameliorate inflammation and AH in SCD patients. It is pertinent to mention that zileuton is structurally similar to hydroxyurea, has been shown to increase hemoglobin F production in primary erythroid cells in vitro, and may have clinical utility in the treatment of SCD.
Acknowledgements

We thank Dr. Timothy Bigby (VA Hospital San Diego, La Jolla, CA) for kindly providing FLAP promoter constructs. This work was supported by National Institute of Health Grant HL-070595 (CSCC) and R01-HL-079916. We thank Institutional core of USC Research Center for Liver Disease for the use of spectrofluorometer and sequence detection instrument (NIH-P30-DK 048522).

Author contributions

NP, Performed most of the experiments, contributed to experimental design, analyzed the data and contributed to the writing of the manuscript.

CG, Performed experiments on LT release

MY, Obtained patient samples, isolated MNC for qRT-PCR

PM, Contributed to study design and hypothesis, obtained sickle samples and wrote the manuscript

VK, Contributed to the hypothesis, study design and experiments, preparation and wrote the manuscript

Disclosures

The authors have no financial conflict of interest.
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Figure Legends

Figure 1. 5-LO and FLAP mRNA expression in MNC, PBM and THP-1. (A and B), quantitative RT-PCR analysis of mRNA in MNC isolated from SCD subjects at steady state (n=9) and healthy controls (n=9). Each data point represents ∆Ct values of 5-LO and FLAP expression from individual subjects. Mean values are represented as black bars. Error bars represent SEM. (C), ∆∆Ct (RQ) values show fold expression of 5-LO and FLAP mRNA in SCD compared to healthy controls. (D), RPA analysis of FLAP, HIF-1α and GAPDH in PIGF treated THP-1 cells and PBM for the indicated time periods. The data is representative of three independent experiments. Where indicated, the vertical lines show repositioned gel lanes.

Figure 2. PIGF induced LTE₄ release, FLAP mRNA expression and ROS formation. (A) PBM or THP-1 cells were treated with PIGF (250 ng/mL) for 24hr and (B) THP-1 cells were pretreated for 30 min with Ab-VEGFR1 (2 µg/mL), LY294002 (10 µM), DPI (10 µM), MK866 (10 µM) and U73122 (10 µM) followed by treatment with PIGF for 24hr. The supernatants were collected and assayed for LTE₄ release by ELISA. (C) RPA analysis of total RNA isolated from THP-1 cells pretreated for 30 min with Ab-VEGFR1, LY294002, DPI, R59949 (10 µM), PDTC (10 µM), SB203580 (10 µM) and PD98059 (10 µM) prior to PIGF treatment for 24hr. (D) THP-1 cells were loaded with DCFH-DA dye for 30 min, incubated with indicated inhibitors for an additional 30 min and treated with PIGF for 4hr. The cells were lysed and the fluorescence was measured. Data are expressed as means ± SEM of three independent experiments. *** P<0.001, ** P<0.01, ns, P> 0.05. Where indicated, the vertical lines show repositioned gel lanes.

Figure 3. PIGF increases FLAP and HIF-1α protein in THP-1 cells. THP-1 cells were pretreated for 30 min with LY294002 and DPI followed by PIGF treatment for indicated time periods. (A) Cytosolic proteins were subjected to western blotting using antibody to FLAP. The same membrane was reprobed with β-actin antibody to normalize protein loading. (B) Nuclear
extracts were subjected to western blotting using antibody to HIF-1α. The same membrane was re-probed with HIF-1β antibody to normalize the protein loading. Proteins were visualized by enhanced chemiluminescence corresponding to their expected molecular weights: FLAP (18 kDa), β-actin (42 kDa), HIF-1α (120 kDa) and HIF-1β (95 kDa). Data are representative of three independent experiments.

**Figure 4. PIGF mediated FLAP and LTE₄ expression involves HIF-1α.** THP-1 cells were transfected with indicated siRNA constructs or expression plasmids, followed by PIGF treatment for 24hr. (A) RPA (B) LTE₄ release and (C) Western blot of nuclear extract using HIF-1α antibody. (D) Western blot of cytosolic extracts from THP-1 cells treated with PIGF for indicated time period (6-24hr) using PHD-2 antibody. Data are representative of three independent experiments. Data are expressed as means ± SEM of three independent experiments. *** P<0.001, ** P<0.01, ns, P> 0.05. Where indicated, the vertical lines show repositioned gel lanes.

**Figure 5. PIGF augments HRE-Luc and FLAP-Luc promoter via activation of PI-3 kinase and HIF-1α.** THP-1 cells co-transfected with HRE-Luc and β-galactosidase plasmid were (A) either pretreated with LY294002 or co-transfected with PTEN and (B) co-transfected with indicated plasmids prior to treatment with PIGF for 24hr. (C) Deletion analysis of FLAP promoter. THP-1 cells were co-transfected with indicated deletion construct and β-galactosidase plasmid, followed by PIGF treatment for 24hr. (D) Schematics of FLAP promoter (-371bp) indicating the location of HIF-1α, NF-κB and C/EBP binding sites. (E and F), PIGF augments minimal FLAP promoter activity through HREs but not NF-κB in THP-1 (E) and PBM (F). THP-1 cells or PBM were co-transfected with indicated promoter constructs and β-galactosidase plasmid, followed by PIGF treatment for 24hr. (G) THP-1 cells were treated with either indicated pharmacological inhibitors or transfected with siRNA or HIF expression plasmids. These cells were then co-transfected with -371FLAP-Luc and β-galactosidase plasmid, followed by PIGF
treatment for 24hr. Luciferase and β-galactosidase activities were measured as described in “Materials and methods”. The luciferase activity was normalized to that of the promoter less pGL3 basic vector. Data are expressed as mean±SEM of three independent experiments. *** P< 0.001, ** P<0.01, ns, P> 0.05.

Figure 6. PIGF augments HIF-1α binding to FLAP promoter in vitro (EMSA) and in vivo (ChIP). (A), Nuclear extracts from THP-1 cells (10 µg) were incubated with a biotinylated double stranded oligonucleotide corresponding to the region (-179 to -159bp) of the FLAP promoter containing the proximal HRE located at -170 to -167bp. Where indicated 50 fold excess of unlabeled wild type probe (lane 3) or antibody to HIF-1α (lane 4) was added. EMSA analysis was also performed with a probe containing a mutation of the HRE (-170bp to -167bp, lane 5). * denotes supershifted band. Data are representative of two independent experiments. (B), THP-1 cells were pretreated with indicated pharmacological inhibitors prior to PIGF stimulation for 4hr. The soluble chromatin was isolated and immunoprecipitated with either HIF-1α antibody (top panel) or control rabbit IgG (lower panel). The primers used to amplify the products flanking HIF-1α binding sites in the FLAP promoter are indicated in Table 1. The middle panel represents the amplification of input DNA before immunoprecipitation. Data are representative of two independent experiments. Where indicated, the vertical lines show repositioned gel lanes.
### Table 1. Oligonucleotide primers used in this study

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<th>Reverse sequence</th>
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Abbreviations: ChIP, Chromatin immuno-precipitation; EMSA, Electrophoretic mobility shift assay; PCR, Polymerase chain reaction; SDM, Site directed mutagenesis. The specific mutations are highlighted as bold and underlined.
Figures

Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1D

Figure 1
Figure 2

**Figure 2A**

![Bar chart showing LTE4 release (pg/106 cells) with PIGF and THP-1](chart)

**Figure 2B**

![Bar chart showing LTE release (% of control) with PIGF and THP-1](chart)

**Figure 2C**

![Western blots for FLAP, HIF-1α, and GAPDH with PIGF, Ab-VEGFR1, LY294002, DPI, R59949, PDTC, SB203580, and PD08053](blots)

**Figure 2D**

![Bar chart showing DCF Fluorescence (% of control) with PIGF, Ab-VEGFR1, DPI, LY294002, and SB203580](chart)
Figure 3

Fig. 3A

Time (h) 0 6 12 24 LY294002 DPI

FLAP

β-Actin

PIGF 24 h

Fig. 3B

Time (h) 0 6 12 24 LY294002 DPI

HIF-1α

HIF-1β
Figure 4

### Fig. 4A

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### Fig. 4B

**Bar graph showing LTE₃ release (pg/10⁶ cells)**

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### Fig. 4C

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### Fig. 4D

**Western Blotting**

- PHD-2
- β-Actin

**Table showing time points**

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Figure 5

Fig. 5A

Fig. 5B

Fig. 5C

Fig. 5D

Figure 5
Figure 5

Fig. 5E

Fig. 5F

Fig. 5G

Figure 5
Figure 6
Placenta growth factor induces 5-lipoxygenase activating protein to increase leukotriene formation in sickle cell disease

Nitin Patel, Caryn S Gonsalves, Minyang Yang, Punam Malik and Vijay K Kalra